tissue extracts. Because of the protective effect of a high concentration of polymers and potassium phosphate, the native structure of the proteins is preserved at room temperature during separation, and the support-free partitioning eliminates sample loss and deactivation of enzymes which is often caused by using the solid support in conventional chromatography. We expect that these merits of the method will apply in the purification of other enzymes.

Conclusion

The capability of the cross-axis CPCs for performing CCC has been demonstrated in the separation and purification of proteins. The unique feature of the apparatus is that it provides sufficient retention of the stationary phase for viscous, low interfacial tension polar solvent systems, such as aqueous-aqueous polymer phase systems. Consequently, the method can be utilized for the fractionation of a wide variety of proteins without adsorptive sample loss and denaturation of proteins caused by the solid support. The CCC method may be further extended to the purification and fractionation of other biopolymers.

See Colour Plate 116.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **Appendix 1: Essential Guides for Isolation/Purification of Enzymes and Proteins; Essential Guides for Isolation/Purification of Immunoglobulins.**

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Ion Exchange

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Introduction

Proteins are polymers of amino acids, the so-called 'building blocks of nature' and are found in all living matter be it of animal, microbial or vegetable origin. By their very structure proteins have an electrical charge and can therefore be fractionated by ion exchange processes. This paper briefly reviews the principles underlying protein purification by ion exchange and addresses some of the process issues associated with their purification.

Proteins

As living cells reproduce, genetic material is passed from parent cells to daughter cells in the form of DNA. DNA is a template coding for the various proteins required for the developing organism. As the organism grows, cells differentiate to form the various organs of the mature organism. Each cell has the capability to express every single protein of the organism, but in life only a small fraction of proteins are expressed. For example muscle cells produce actin and myosin to facilitate movement, the pancreas produces chymotrypsinogen and trypsinogen to facilitate digestion and lymphocytes are responsible for the expression of immunoglobulins which provide immunity from infection and disease.

Because of their functional and structural roles in nature, proteins have significant commercial potential in many areas including food and beverage, biological detergents, diagnostic enzymes, veterinary, agricultural and pharmaceutical applications. However, because of their diversity, the challenges of their purification are immense and their isolation from a particular tissue or organ, regardless of host, may be regarded as 'searching for a needle in a haystack'.

Protein Structure

Proteins are polymers of amino acids bonded together through amide linkages. There exist 20 common amino acids in nature ranging in molecular mass from 75 to just over 200 Da. Proteins range in molecular mass from around 10 000 up to $> 1\,000\,000$ Da, and consequently their amino acid sequence or primary sequence may be hundreds of residues in length. Of the 20 amino acids, several have positively or negatively charged side chains, while others have neutral side chains, which may have hydrophilic or hydrophobic properties. The primary sequence of a protein results in a zwitterion with the positively charged *N* terminus balancing the negatively charged *C* terminus. However, the charges of the side chains of the charged amino acids and the pK_a values of their functional groups give, at least in principle, an overall positive or negative charge at a given pH. However, proteins are not simple structures and certain sequences of amino acids fold to give secondary structures such as helices and pleated sheets. This secondary structure scrambles up to give a three-dimensional tertiary structure. Some proteins exist as an assembly of subunits giving a quaternary structure. Many proteins are glycosylated to aid with molecular recognition *in vivo* and this influences their shape and surface properties.

The net effect of the three-dimensional structure of proteins is that their theoretical charge or hydrophobicity based on a primary sequence bears little relation to the actual properties of the molecule in its native state. If, for example, all the charged groups are buried inside a pocket in the molecule, then its response to an ion exchanger may be quite weak. The three-dimensional structure of a protein is associated with function and for the purified protein to have intrinsic value, its three-dimensional structure should be retained. This presents practical difficulties in terms of purification, because a denatured protein may not readily, if at all, refold back to its native state. For mammalian systems, typical physiological conditions are pH 7.4 and 0.15 M NaCl and most proteins would be stable and active under these conditions. However, deviations in operating pH and, to a lesser extent, ionic strength, may irreversibly denature the protein of interest, which can severely restrict the mode of purification available to the chromatographer.

Methods of Protein Purification

Prior to carrying out any practical studies, the protein chemist is provided with a range of chromatographic techniques, the use of which should enable effective purification to be achieved. Those techniques suitable for low pressure operation include those listed in **Table 1**. While all of these techniques are suitable for laboratory-scale use, those typically scaled-up include ion exchange, hydrophobic interaction, affinity and size exclusion. These techniques each exploit differing physicochemical properties of the protein molecules as manifest by their three-dimensional structure. Ion exchange chromatography and hydrophobic interaction chromatography rely on electrostatic interactions between a charged stationary phase and charged surfaces of the protein or hydrophobic interactions between a hydrophobic stationary phase and hydrophobic surfaces of the protein respectively. Affinity separations rely on a biospecific interaction, for example the interaction of an enzyme with its immobilized substrate or an immunoglobulin with its immobilized antigen. Size exclusion chromatography is a molecular sieving effected by the three-dimensional size and shape of the protein. One or more of these techniques should be suitable for protein purification with their choice influenced by the selectivity requirements of the process in terms of both the target and contaminants.

Ionic Properties of Proteins

For the reasons described above, all proteins will have ionic properties, and their three-dimensional structure imparts a subtle uniqueness to their ionic charge, which is available for exploitation by ion exchange chromatography during their purification. In a similar manner to small molecules, such as organic acids, which vary their charge with pH, as prescribed by their pK_a, proteins have an isoelectric point or p*I*. The

Table 1 Techniques available for low pressure chromatography of proteins

Salt precipitation
Ion exchange
Size exclusion
Hydrophobic interaction
Thiophilic interaction
Affinity
Chiral

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p*I* of a protein is the pH where it has a net charge of zero.

When the pH is greater than the p*I*, the protein will have a net negative charge and may bind to an anion exchanger, provided that the pH is less than the pK_a of the functional group. If the pH is less than the p*I* the protein will have a net positive charge and may bind to a cation exchanger, provided the pH is greater than the pK_a of the functional group. As a rough guide most proteins have a p*I* of less than 7, ranging typically from 4.5 to 6.5. The major exceptions are the immunoglobulins which typically have a p*I* of greater than 7.

Ion Exchange Chromatography of Proteins

Principles

On the basis of the physicochemical issues discussed above, it might appear that by following a few simple rules, centred around pH, an ion exchange separation can be designed. The first barrier to overcome is to find out the p*I* of the protein. Unless the protein is well characterized, this may not be documented, and although it can be readily determined by techniques such as isoelectric focusing, this presupposes that it can be obtained in a relatively pure state. A second barrier is the pH and ionic strength stability ranges of the protein. This can be determined readily by a parametric study, centred around a robust assay for the protein, typically linked to its biological function. A third, and often underestimated barrier, is the influence of the other contaminants within the feedstream and how they may impact on the efficiency of the ion exchange separation. For example, competitive adsorption of an unwanted contaminant to the adsorbent can significantly hamper the selectivity of the separation and the process economics. Other key considerations include the mobile phase composition as defined by the preceding chromatographic step and its impact on an ion exchange separation and the eluent composition and its impact on the subsequent downstream step.

Method Scouting

In the light of the issues highlighted above, the chromatographer can start to develop the ion exchange process. A broad strategy for ion exchange is represented in **Figure 1.** Intuitively, it would seem reasonable to expect ion exchange steps to be of the positive form, whereby the target is retained by the exchanger, and assuming elution volume is less than feedstock volume, has the potential to effect product concentration. Such an approach may be preferable if

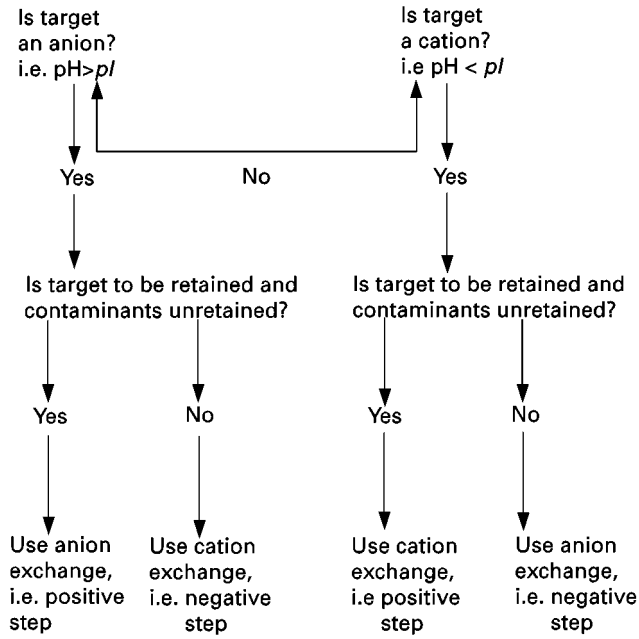
Is target an anion? i.e. pH>pI
Is target a cation? i.e pH < pI
Yes
No
Yes
Is target to be retained and contaminants unretained?
Is target to be retained and contaminants unretained?
Yes
No
Yes
No
Use anion exchange, i.e. positive step
Use cation exchange, i.e. negative step
Use cation exchange, i.e positive step
Use anion exchange, i.e. negative step

Figure 1 Approaches to development of an ion exchange chromatographic process. Copyright © 1998 Whatman International Ltd., reproduced with permission.

the target binds more strongly to the exchanger than the contaminants, so they are displaced by the target during loading. The isolation of ovalbumin from hen egg-white by anion exchange chromatography is one such example (**Figure 2**), where we have shown ovalbumin to displace the less anionic conalbumin component during adsorption. However, the desorbed protein product is typically in a mobile phase containing up to 1 M NaCl and this may be unsuitable for adsorption in a subsequent step, for example an affinity interaction. If this is the case then another unit process, such as diafiltration, needs to be introduced which may be costly, time-consuming and could result in additional yield/activity loss.

An approach which may often be dismissed, but in fact can be highly efficient is the negative step. In this case the contaminants bind to the adsorbent and the target passes unretained during loading. Since there is no volume reduction, product concentration remains constant, but purity increases. If the target is present in excess, then a modest adsorbent volume may suffice, which has an impact on cost, but perhaps as important, the mobile phase composition remains unchanged, which may facilitate the subsequent chromatographic step. Negative steps are routinely employed during immunoglobulin isolation from serum or plasma, where the anionic albumin contaminants adsorb to an anion exchanger at neutral pH, while the cationic immunoglobulin fraction passes unretained through the exchanger, as represented in **Figure 3**.

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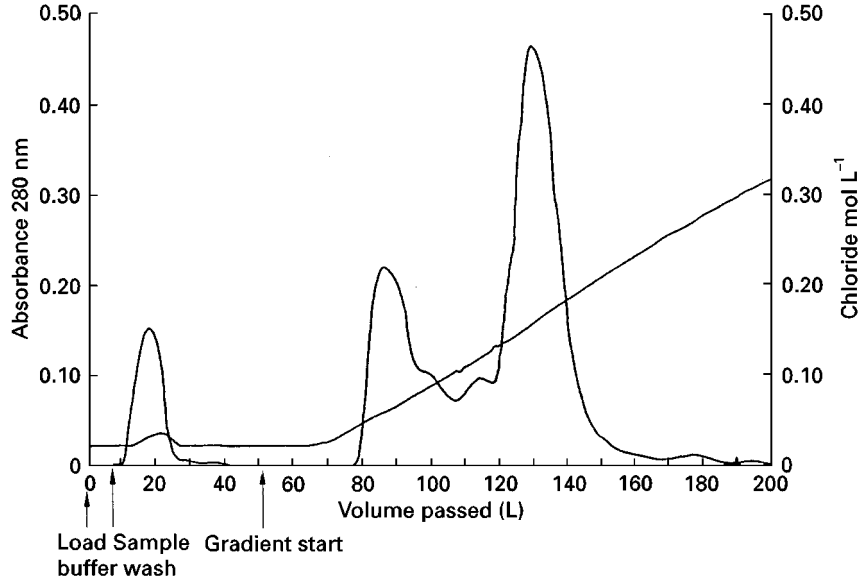

Figure 2 Chromatography of hen egg-white proteins on Whatman DE52 using 0.025 M Tris/HCl buffer, pH 7.5 in a column (45 cm i.d. × 16 cm) at a flow rate of 1 L min^{-1}. Copyright © 1998 Whatman International Ltd., reproduced with permission.

These are some of the fundamental considerations during method scouting and are based on two questions. Firstly, at a given pH will the target bind to an anion exchanger or a cation exchanger? Secondly, is this what is wanted?

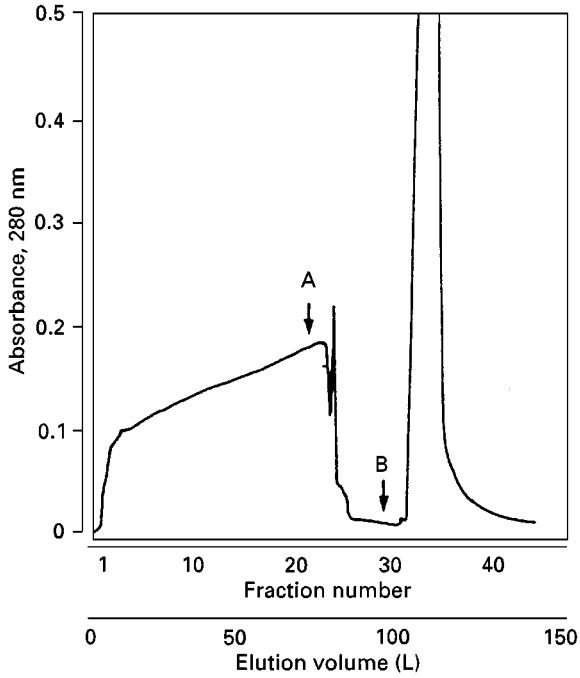

Figure 3 Chromatography of a goat serum preparation on Whatman QA52 using 0.02 M Tris/HCl buffer, pH 7.5 in a column (32.5 cm i.d. × 12 cm). A, denotes a wash in loading buffer; B, denotes a wash using loading buffer containing 0.5 M NaCl. Copyright © 1998 Whatman International Ltd., reproduced with permission.

Given that the mobile phase of the feedstock is determined by the upstream process and any significant adjustments add cost and complexity, the chromatographer can now start to address these two questions. Due to the nature of proteins and the influences of protein : protein interactions which may occur in the feedstream, it is difficult, if not impossible, to predict the optimal chromatographic conditions without practical study. It is therefore common practice to carry out a parametric study, investigating the influence of pH and ionic strength with different ion exchangers. This is potentially a time-consuming process, but one that will aid in process optimization. This traditionally was performed manually, but more recently automated workstations have become commercially available which can be programmed to carry out multivariable parametric studies automatically.

Media Selection

A key consideration during method scouting is which ion exchanger to use. The protein chromatographer is offered a range of strong or weak anion or cation exchangers, from several suppliers. The functional groups are broadly similar across the range, but the base matrices range from polysaccharides including agarose, cellulose and dextran to polymeric supports and advanced composites. Given that each manufacturer has proprietary chemical processes, the offerings available are quite diverse. In order to assess the impact of media selection on method scouting and development, we screened 70 different commercially

available anion and cation exchangers, which may be considered for process-scale protein separations. Each medium was screened under identical conditions. Perhaps not surprisingly, our data demonstrated that 70 different media had 70 different properties. Our data, descriptive rather than prescriptive, suggest that media effectiveness is process dependent rather than supplier dependent and the thought process of 'it worked last time' is not an appropriate rationale for developing a second process using the same adsorbent.

Method Development

When an appropriate ion exchanger and mobile phase system have been identified, it must then be decided whether to conduct the separation in either a column contactor or a batch stirred tank system. The former technique, being contained, lends itself to automation and control, but the latter technique, albeit classical, should not be dismissed. If, for example, the feedstream/adsorbent volume ratio is high, perhaps $> 20:1$, then the time to pump the feedstream through a packed bed of adsorbent would be several hours. A batch stirred tank adsorption including medium collection by centrifugation should take less than 1 h. Similarly in a large scale process where several hundreds of kilograms of ion exchanger are used, columns are costly and often cumbersome to use, so a large batch system may be preferred due to its simplicity.

For highly selective separations where desorption and elution conditions are critical then a column-based technique would be appropriate, typically using gradient elution.

As stated earlier, proteins are large molecules with a size up to hundreds of angstroms and consequently diffusion into and out of the pores of an ion exchanger is the rate limiting step both in terms of capture efficiency during adsorption and selectivity during desorption and elution. In order to enhance each of these parameters, one needs to maximize residence time of the adsorbate with the absorbent to increase the capture efficiency of the target protein and in order to maximize selectivity, one needs to provide adequate time for the desorbed protein to diffuse into the bulk liquid phase, so it elutes as a sharp peak. Flow rate is clearly the critical factor to regulate these adsorption/desorption rates and this equates with process-time. Unlike ion exchange of small ions, where pore diffusion rates while limiting have minimal criticality, they are crucial for effective ion exchange chromatography of proteins and must be considered during methods development. Typical linear flow rates for polysaccharide-based ion exchangers would be 30–300 cm h^{-1} and for advanced polymeric-based media, a further 10-fold increase in flow may be possible. However, it should be noted that the maximum flow rate specification of the ion exchanger and an operational flow rate for effective protein binding and elution may be widely different, and will likely to be determined by the nature of the protein separation itself.

Scale-down Studies

Having defined the ion exchanger, the mobile phases and the mode of operation, a series of small scale studies will be carried out at the laboratory bench to assess process economics and perhaps to carry out sensitivity analysis and validation support studies.

Scaled-down studies are very valuable and enable a substantial amount of data to be generated and collated in a cost-effective manner, although the timescale may be similar to that required for large scale work. The key feature of a small scale study is that the contactor is a scaled-down version of the process system. For a batch process the aspect ratio of the tank and tip speed of the agitator, etc., would be the same for both scales of operation. The ratio of feedstock volume to mass of ion exchanger would be constant as would the contact times for adsorption, washing and elution. For a column separation, column bed height would be identical at both scales of operation and linear flow rate would be maintained throughout the process. Provided that all mobile phase volumes used were in proportion to the amount of ion exchanger used, and that feedstock and buffer compositions remain unaltered, a small scale study should be a good model of the large scale process separation. In the anion exchange chromatography of hen egg-white proteins, for example, we have reported the 1000-fold scale-up of a column process from a 25 mL column to a 25 L column.

Process validation is a critical area in the isolation of therapeutic proteins. In these applications it is crucial that for multiple uses of the adsorbent, the eluting fraction containing the target protein has a consistent composition from run to run and that it meets a specification in terms of microbial bioburden, endotoxin levels, pyrogenicity and viral contamination. These aspects of process validation have been adequately reviewed by Sofer and Nystrom.

A widely used mobile phase for regeneration of ion exchangers following protein chromatography is sodium hydroxide. It is well established that exposure of a column of ion exchanger to 0.5–2.0 M NaOH for up to 12 h is an effective means of regenerating the medium. Importantly, these conditions are also acknowledged to be effective at sanitization of the ion exchange system, and we have confirmed this to be

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the case following gross microbial contamination of columns of both anion and cation exchangers.

A key element of process validation is the chemical stability of the ion exchanger to the cleaning/sanitizing regent systems. We have developed protocols for monitoring hydrolysis of functional groups, referred to as ligand leakage, and also matrix dissolution, in order to address these concerns.

These process validation studies are typically conducted in scale-down mode, and confirmatory checks made subsequently at process scale.

Process Scale Ion Exchange Chromatography of Proteins

Having defined the feedstock and mobile phases, selected the ion exchanger and selected a batch or column mode of operation, the chromatographer should find himself or herself in a position to scale-up the process.

It is difficult to predict cost information on process scale ion exchange separations since much depends on the upstream and downstream process requirements and reusability of the ion exchanger, etc. We have reported a cost estimate for single usage of 25 kg of DE52 in batch and column operation (see Ganetsos and Barker), but this is exemplary only. Unfortunately information of this type, while in existence, is proprietary and therefore withheld.

Industry has carried out large scale ion exchange chromatography of proteins for the last few decades and with the established principles described above, there is no reason to assume that things will change significantly over the short to medium term. In the longer term, developments in fluidized/expanded beds and membrane adsorbers may offer new opportunities in this area of chromatography.

See also: **II/Affinity Separation:** Rational Design, Synthesis and Evaluation: Affinity Ligands. **III/Proteins:** Centrifugation; Electrophoresis; High-Speed Countercurrent Chromatography.

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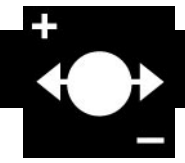
Metalloprotiens: Chromatography

See **III / METALLOPROTEINS: CHROMATOGRAPHY**

Thin-Layer (Planar) Chromatography

See **III / PEPTIDES AND PROTEINS: Thin-Layer (Planar) Chromatography**

PROTEOMICS: ELECTROPHORESIS



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Introduction

The first complete genome sequence, that of *Haemophilus influenzae*, was published in 1995. Intense effort over the last three years has resulted in the completion of the genomes for a further 12 micro-organisms